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Virus Particles from Conidia of *Penicillium* Species

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Virus particles and their component double-stranded ribonucleic acid (dsRNA) have been isolated from conidia and mycelia of certain *Penicillium* species. The conidia and mycelia of *P. stoloniferum* NRRL 5267 contained 75 and 85 μg of dsRNA/g (dry weight), respectively. Of the total dsRNA released from NRRL 5267 conidia, 10% was nonencapsulated. Conidia of *P. brevi-compactum* NRRL 5260 and *P. chrysogenum* Q-176 contained 2 and 120 μg of dsRNA/g (dry weight), respectively, whereas mycelium from the two species contained 3 and 95 μg of dsRNA/g (dry weight), respectively. No viruses were isolated from conidia or mycelia of *P. stoloniferum* NRRL 859. A method is described for disruption of both conidia and mycelia. The technique facilitates the isolation and characterization of fungal viruses and their component dsRNA and also potentiates surveying of fungal isolates for the presence of virus.

Polyhedral virus particles have been reported in a number of fungal species (5, 12). Ellis and Kleinschmidt (11) were the first to present evidence that viruses occur in *Penicillium stoloniferum*. Banks et al. (3) isolated and characterized viruses and their component double-stranded ribonucleic acid (dsRNA) from *P. stoloniferum*. Bozarth et al. (6) described two serologically distinct viruses having different electrophoretic mobilities from *P. stoloniferum* NRRL 5267. Wood et al. (20) isolated a virus from *P. brevi-compactum* and demonstrated the double-stranded nature of its component RNA. The component dsRNA of a virus from a penicillin-producing strain of *P. chrysogenum* was characterized by Lemke and Ness (14).

Viruses have been observed throughout the cytoplasm in thin sections of conidiospores of *P. stoloniferum* and *P. brevi-compactum* (13), of mushroom basidiospores (10), and of the zoospores of *Plasmidiophora* (1). However, the isolation and biochemical characterization of virus from fungal conidia have not been reported.

We have isolated and characterized viruses and their component dsRNA from conidia of *P. stoloniferum* NRRL 5267, *P. brevi-compactum* NRRL 5260, and *P. chrysogenum* Q-176. Although Banks et al. (4) have described an efficient pilot plant-scale isolation of viral dsRNA from several species of *Penicillium* and *Aspergillus* and Moffitt and Lister (17) have recently reported a rapid method for surveying

fungal isolates for dsRNA-containing viruses, we developed a method for both the detection and isolation of viruses and dsRNA from sub-gram quantities of conidia and mycelia. The release of macromolecules, with time of disruption of conidia, provides the information necessary for quantitatively comparing concentrations of viral dsRNA in conidia and mycelia. The comparison provides an insight into the possible mode of transmission and rate of replication of viruses from the conidial to the hyphal stages of growth.

MATERIALS AND METHODS

Production and harvest of conidia. Cultures of *P. stoloniferum* NRRL 5267, *P. brevi-compactum* NRRL 5260, *P. chrysogenum* Q-176, and *P. stoloniferum* NRRL 859 were furnished by the Agriculture Research Service Culture Collection, Northern Regional Research Laboratory. These cultures were maintained on potato-dextrose agar (PDA). Conidia of the organisms were produced by the method of Sansing and Ciegler (unpublished), as follows. White bread, containing no preservatives, was cut into 1.5-cm cubes; 200 g of the cubed bread was placed in a 2.8-liter Fernbach flask and autoclaved for 15 min. The bread was inoculated with 20 ml of a conidial suspension from PDA slant cultures and incubated for 10 days at 28 C. Conidia were harvested from the bread by adding 800 ml of a 10⁻⁴% solution of Triton X-100 and shaking the flask, after which the conidial suspension was filtered through cheesecloth to remove the bread cubes and the suspension was filtered through glass wool to remove bread fines. Conidia were pelleted by centrifugation

at $2,000 \times g$ for 15 min. The conidial pellet was suspended in 0.1 M potassium phosphate buffer, pH 7.2, and recentrifuged. This step was repeated twice to remove any starch particles remaining from the bread.

Disruption of conidia. Known concentrations of conidia (as given under individual sections) were suspended in 0.1 M phosphate buffer (pH 7.2) and added to 75-ml glass Bronwill cell homogenizer flasks, each containing 45 g of 0.5-mm glass beads. Each sample was homogenized for a specific time (see assay sections) in a Bronwill mechanical cell homogenizer (Braun model MSK) at 4000 rpm under a cold CO_2 stream. All homogenizing flask temperatures were maintained at 0 to 1 C. Samples of homogenates were examined microscopically to determine the percentage disruption of conidia.

Disruption of mycelia. A 10% sucrose-2% yeast extract medium was inoculated with a conidial suspension and incubated at 28 C on a Brunswick shaker at 250 rpm in 2.8-liter Fernbach flasks (500 ml of medium) for 72 h. Mycelia were harvested by vacuum filtration and then suspended in 0.1 M phosphate buffer, pH 7.2 (5 ml of buffer/g [wet weight] of mycelium). The suspension was added to 75-ml Bronwill flasks containing 45 g of 1.0-mm glass beads and homogenized for 3 min at 4000 rpm under a CO_2 stream.

Detection of virus. The homogenates resulting from the disruption of 1.5 g (dry weight) of conidia of each of the four *Penicillium* strains were centrifuged at $2,000 \times g$ (10 min) to remove cell debris as described in Fig. 1. The supernatant fluid (SNF) was centrifuged at $105,000 \times g$ for 2.5 h. The resulting pellet was suspended in 2 ml of 0.1 M phosphate buffer, pH 7.2, and centrifuged at $4,000 \times g$ for 10 min. The supernatant was filtered through a 0.45- μm membrane filter (Millipore Corp.). The virus preparation was applied to carbon-coated Formvar grids, stained with 0.5% uranyl acetate, and rinsed twice with distilled water. The grids were examined by electron microscopy (RCA EMU-3 electron microscope) at an instrument magnification of $\times 32,000$. The viruses were analyzed by polyacrylamide gel electrophoresis on 2.4% gels for 5 h (6 mA per tube) at 25 C as described by Loening and Ingle (15). Gels were scanned at 260 nm with a Gilford linear transport system.

Isolation and quantitation of viral dsRNA. As shown in Fig. 1, two volumes of cold methanol were added to the SNF from each conidial homogenate, and the precipitates were sedimented by centrifugation at $2,000 \times g$ for 10 min. Each precipitate was dissolved in 0.2 M sodium acetate and treated with an equal volume of aqueous 90% phenol containing 0.1% (wt/vol) 8-hydroxy quinoline (9). The mixture was shaken for 20 min at 25 C and centrifuged at $4,000 \times g$ for 20 min. Nucleic acid was freed from phenol by repeated precipitation from 0.2 M sodium acetate with equal volumes of cold methanol. The nucleic acid precipitate was dissolved in a minimal volume of 0.15 M NaCl-0.15 M sodium citrate (SSC) solution, pH 7.4.

The RNA samples were incubated with 1.0 μg of ribonuclease B (Sigma Chemical Co.) per ml of 0.3 M

STE buffer (0.3 M NaCl, 0.01 M tris(hydroxymethyl)-aminomethane, and 0.001 M ethylenediaminetetraacetic acid) at 37 C for 30 min. The remaining RNA was precipitated with cold methanol, the mixture was centrifuged at $8,000 \times g$ for 10 min, and the pellet was redissolved in SSC buffer and subjected to electrophoresis. Mobilities of RNA components were compared to those of a purified dsRNA preparation (*P. stoloniferum* NRRL 5267) of known concentration and standard yeast transfer RNA (Sigma Chemical Co.). The dsRNA was measured by integration of the areas under the electrophoretic peaks compared to standards.

Time release of macromolecules. Three 1.5-g (dry weight) samples of *P. stoloniferum* NRRL 5267 conidia were homogenized for 1, 2, and 4 min, respectively, by the previously described method. Three 3.0-g and three 6.0-g samples (dry weight) of conidia were homogenized for the same time intervals. Cell debris was removed from homogenates by centrifugation. The SNF was quantitatively assayed for total RNA, deoxyribonucleic acid (DNA), protein, and trehalase by the following methods.

Quantitation of total RNA, DNA, and protein in conidial homogenates. Supernatants from centrifuged conidial homogenates were assayed for RNA by the orcinol method of Brown (7) and for DNA by the method of Burton (8). The pellet resulting from the hot trichloroacetic acid treatment of homogenates was dissolved in 1 N NaOH by heating at 90 C for 30 min. This solution was quantitatively assayed for protein by the method of Lowry et al. (16).

Determination of trehalase activity. Trehalase activity was assayed by adding 0.1 ml of SNF to test tubes containing 2.0 ml of trehalose (2.0% wt/vol) in 0.1 M potassium phosphate buffer (pH 5.6) and incubating them at 25 C for 1 h. Glucose was assayed by the method of Nelson (19).

Ratio of encapsulated to nonencapsulated dsRNA. Five 3.0-g (dry weight) samples of *P. stoloniferum* NRRL 5267 conidia were homogenized for 2 min. Cell debris was removed, and the resulting SNFs were pooled and centrifuged at $105,000 \times g$ for 2 h to pellet virus particles. The virus-free SNF was quantitatively assayed for dsRNA. The virus pellet was suspended in 10 ml of 0.1 M phosphate buffer (pH 7.2), and 5 ml was assayed for viral dsRNA. The remaining suspension was diluted to 45 ml with buffer and homogenized in a Bronwill flask containing 45 g of 0.5-mm glass beads, as described for disruption of conidia. This homogenate was centrifuged at $105,000 \times g$ for 2 h, after which SNF was assayed for dsRNA.

RESULTS

Detection of virus and dsRNA in conidia. Analyses of conidial homogenates of *P. stoloniferum* NRRL 5267, *P. brevis-compactum* NRRL 5260, and *P. chrysogenum* Q-176 by electron microscopy demonstrated the presence of isometric virus particles similar in size to those reported from mycelia (2, 6, 11, 13, 14, 18, 20). Their electrophoretic mobilities were identical to those determined from mycelial sources

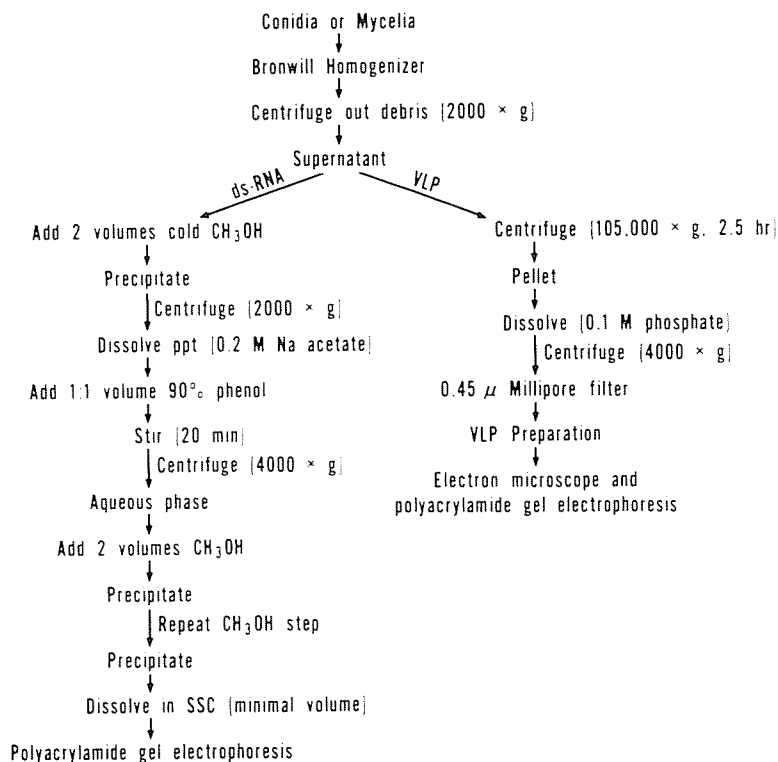


FIG. 1. Flow diagram for the preparation of virus particles and viral double-stranded ribonucleic acid (dsRNA) from conidia and mycelia of *Penicillium* species.

in this investigation. No particles were detected in conidial or mycelial homogenates of strain NRRL 859 of *P. stoloniferum*.

Electrophoretograms of viral dsRNA isolated from conidia of the three *Penicillium* species, after treatment with ribonuclease, are depicted in Fig. 2. The five dsRNA bands observed for the fast- and slow-moving virus species of *P. stoloniferum* are identical to bands observed from mycelial extracts (6). No attempt was made to determine concentrations of the fast- and slow-moving dsRNAs. The virus particles from *P. brevi-compactum* conidia contained the same three dsRNA species reported for mycelial extracts of the organism (20). Similarly, the three distinct bands observed for the virus from conidia of *P. chrysogenum* correspond to those reported for mycelial extracts (18). The three bands of *P. brevi-compactum* have electrophoretic mobilities similar to those for *P. chrysogenum*.

The concentration of viral dsRNA in conidia and mycelia of the three species is compared in Table 1. The amount of viral dsRNA in conidia of *P. chrysogenum* (120 $\mu\text{g/g}$ dry weight) is

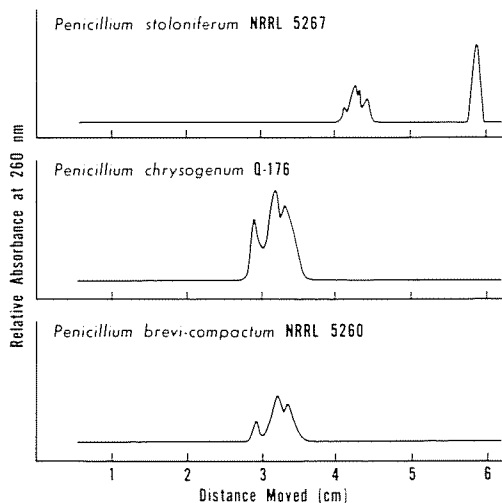


FIG. 2. Electrophoretic profiles (polyacrylamide gel) of dsRNA extracted from conidia of *Penicillium* species. The isolated RNA from the three fungal species were separated electrophoretically on polyacrylamide gels (2.4%) for 3 h at 6 mA/tube.

almost twice that from *P. stoloniferum* (75 $\mu\text{g/g}$ dry weight) and 60 times greater than in *P. brevi-compactum* (2 $\mu\text{g/g}$ dry weight). The concentration of viral dsRNA in the conidia of these species is quite comparable to that found in their mycelial forms.

Time release of macromolecules. The relationship between concentrations of macromolecules (RNA, DNA, and protein), concentrations of conidia, and disruption time is shown in Table 2. Optimal breakage of conidia (88 to 94%) occurred at 4 min. At all conidial concentrations the percent of breakage increased with disruption time. The release of macromolecules also increased with increased disruption time at all three conidial concentrations; maximal release came at the 4-min time intervals. Trehalase activity increased as a

function of disruption time, with maximal activity occurring at 4 min. The amount of dsRNA released from disrupted conidia represents 0.8 to 1.0% of the total RNA released and is 0.003 to 0.004% of the total spore mass on a dry weight basis.

Nonencapsulated dsRNA in conidia. Quantitative analyses of 15 g of conidia (dry weight) from *P. stoloniferum* NRRL 5267 indicated that 10% of the dsRNA exists free in the conidia. Homogenization of virus with subsequent repelleting resulted in no detectable dsRNA in the SNF. This failure to detect nonencapsulated dsRNA indicated that viruses are not being disrupted as a result of mechanical homogenization.

DISCUSSION

This report describes the first isolation and characterization of virus particles from fungal conidia. Electron microscope comparisons of particles isolated from conidia or mycelia of a particular isolate show that particles from either source are structurally identical. Biochemical and physical comparisons of mycoviral dsRNA isolated from conidia or from mycelia of a particular isolate show that the dsRNA is qualitatively the same. Quantitatively, the ratio of viral dsRNA per gram (dry weight) of conidia is equal to the ratio of viral dsRNA per gram (dry weight) of mycelia.

The presence of virus in conidia explains a mechanism for viral sustenance during non-vegetative stages of the life cycle of fungal isolates.

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TABLE 1. *Relative concentrations of viral double-stranded ribonucleic acid (dsRNA) in conidia and mycelia of Penicillium species*

Organism	dsRNA	
	Conidia ^a ($\mu\text{g/g}$ dry wt)	Mycelia ^b ($\mu\text{g/g}$ dry wt)
<i>P. stoloniferum</i> NRRL 5267	75	85
<i>P. brevi-compactum</i> NRRL 5260	2	3
<i>P. chrysogenum</i> Q-176 ...	120	95

^a These values were obtained by analyses of RNA extracted from 1.5 g (dry wt) of conidia of NRRL 5267 and Q-176, and 15 g (dry wt) of conidia of NRRL 5260.

^b These values were obtained by analyses of RNA extracted from mycelia harvested after 72 h of growth at 28 C as a shake culture.

TABLE 2. *The time release of macromolecules from conidia of Penicillium stoloniferum* NRRL 5267 by mechanical disruption

Treatment		Wet wt of conidia (g)	Total				Trehalase activity	Breakage of conidia (%)
Dry wt of conidia (g)	Disruption time (min)		dsRNA	RNA	DNA	Protein		
1.5	1	5	33 ^a	3.4 ^b	0.4 ^b	132 ^b	330 ^c	68
1.5	2	5	52	5.6	1.4	165	420	82
1.5	4	5	62	6.4	1.5	195	490	91
3.0	1	10	34	4.9	0.8	162	740	51
3.0	2	10	44	5.4	0.9	185	930	79
3.0	4	10	55	6.8	1.4	237	1,050	94
6.0	1	20	26	4.8	0.8	157	1,690	61
6.0	2	20	40	5.2	1.0	199	2,110	81
6.0	4	20	52	5.5	1.1	239	2,270	88

^a Micrograms per gram (dry weight) of conidia.

^b Milligrams per gram (dry weight) of conidia.

^c Micrograms of glucose released per hour per milligram of homogenate.

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